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ANTIPSORIATIC COMPOSITIONS, METHOD OF MAKING, AND METHOD OF USING

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BACKGROUND OF INVENTION

1. Field of the Invention

The present invention relates to skin treating compositions, to a method of making the compositions, and to a method of using the compositions in the treatment of skin. In another aspect, the present invention relates to antipsoriasis compositions, to a method of making the compositions, and to a method of using the compositions to treat psoriasis. In even another aspect, the present invention relates to botanical-derived skin treating compositions, to a method of making the compositions, and to a method of using the compositions to treat skin.

2. Description of the Related Art

Psoriasis is a chronic skin condition characterized by itchy, flaky skin. It is estimated that two percent of the United States population, more than four million people, will suffer from psoriasis during their lives. Psoriasis conditions can range from mild to severe.

In the United States, between about 150,000 and 250,000 new cases of psoriasis occur each year, with about 40,000 of these cases classified as severe. Sufferers of psoriasis must endure not only the irritating disease itself, but also the embarrassment of skin disfigurement.

The total annual cost for treating psoriasis on an outpatient basis is estimated at more than \$1.5 billion. It is estimated that psoriasis sufferers are spending an average of \$500 per year on psoriasis treatment to achieve only temporary relief. Severe cases that require hospitalization may require an expenditure of up to \$10,000.

The compound 3-methylanthalin has long been utilized in the treatment of psoriasis, and is listed in the *Merck Index* as an antipsoriatic. Chrysarobin is a mixture of compounds derived from Goa powder, and includes 3-methylanthalin. Goa powder itself is derived from the wood and bark of *Andria Araroba Aguiar* (Fam. Leguminosae). Literature references describing the isolation of and structure of Chrysarobin date back to the early 1800s. A method of reducing Chrysarobin to obtain 3-methylanthalin was known as early as 1931.

Known psoriasis treatments include: antimetabolites such as methotrexate; corticosteroids such as triamcinolone creams or injection, clobetasol propionate cream, and hydrocortisone; keratolytic/destructive agents such as anthralin or salicylic acid; lubricants such as hydrogenated vegetable oils and white petroleum; oral retinoids such as etretinate or isotretinoin tablets; photochemotherapy such as methoxsalen or trioxsalen capsules, and coal tar; and topical cholecalciferol analogs such as calcipotriene ointment, a topical vitamin D₃, known commercially as Dovonex® (Squibb, Buffalo, N.Y.).

Numerous botanical protocols for the treatment of psoriasis are known, including the use of extracts of various herbs, roots, seeds, flowers, berries, and twigs. See *Therapeutic Botanical Protocol for Psoriasis*, Protocol Journal of Botanical Medicines, Aug. 1994, pp. 1-38.

However, the known psoriasis treatments suffer from one or more deficiencies, including potential toxic side effects and achieving only temporary relief. Thus there is a need for improved psoriasis treatment.

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SUMMARY OF INVENTION

It is an object of the present invention to provide a composition and method of treating psoriasis.

It is another object of the present invention to provide a method for making 3-methylanthalin.

It is even another object of the present invention to provide a method of making chrysophanol.

It is still another object of the present invention to provide a method of making aloe-emodin.

It is a further object to provide a method of making aloe-emodin monoacetate.

It is an additional object to provide a composition extracted from a botanical specimen that has efficacy in treating psoriasis.

It is another object to provide such a composition having a plurality of polyphenols therein.

It is a further object to provide a method of treating psoriasis with such a composition.

It is yet another object to provide a method of extracting such a composition from the botanical specimen.

These and other objects of the present invention are achieved by the composition and methods of the present invention.

According to even still another embodiment of the present invention there is provided a method of making a product comprising at least one of 3-methylanthalin, chrysophanol, aloe-emodin, and aloe-emodin monoacetate comprising contacting root of the plant *Asphodelus Microcarpus* with acetic acid to form the product. A further embodiment of the method includes recovering 3-methylanthalin, chrysophanol, aloe-emodin, or aloe-emodin monoacetate from the product. Any of the products may be further derivatized.

According to yet even another embodiment of the present invention there is provided a method of obtaining either 3-methylanthalin, chrysophanol, or aloe-emodin by recovery of the desired compound from the root of the plant *Asphodelus Microcarpus*. The polyphenols may be further derivatized.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of the analytical fractionation scheme utilized in Example 4.

FIG. 2 shown the peak assignments used to generate the data of Table 3 for the XAD-2 resin DCM eluate fraction 23.

FIGS. 3 and 4, show, respectively, the direct probe (DP) mass spectrometry data and liquid secondary ion mass spectrometry (LSIMS) data pertaining to the XAD-2 resin DCM eluate fraction 23 of FIG. 1.

FIGS. 5, 6, and 7 show, respectively, a GC-MS chromatogram, DP data, and LSIMS data for the chemical composition of XAD-2 resin MeOH eluate DCM-insoluble fraction 26 of FIG. 1.

FIGS. 8, 9, and 10 show, respectively, a GC-MS chromatogram, DP data, and LSIMS data for the hydrolyzed XAD-2 resin MeOH eluate DCM-insoluble fraction 29 of FIG. 1.

FIGS. 11, 12, and 13 show, respectively, a GC-MS chromatogram, DP data, and LSIMS data for carboxylic acid fraction 28.

FIG. 14 shows GC-MS chromatogram data from the methylated carboxylic acids of Example 5.

FIGS. 15, 16, and 17 show, respectively, a GC-MS chromatogram, DP data, and LSIMS data for the neutral/phenol fraction 36 of FIG. 1.

FIGS. 18–23 show, respectively, GC-MS chromatograms, DP data, and LSIMS data for the neutral/phenol fraction DCM silica gel eluate and the neutral/phenol fraction MeOH silica gel eluate.

FIG. 24 is a plot showing the effects of the treatment solution of Example 2 on HNF in fibroblasts.

DETAILED DESCRIPTION OF THE INVENTION

In the practice of the present invention, a composition is made from the plant *Asphodelus Microcarpus* that comprises compounds useful in the treatment of mammalian skin disorders, especially psoriasis. *Asphodelus Microcarpus* is a plant that is native to the Middle East; specifically, it can be easily found in the Northern regions of Israel, as well as in regions from the Canary Islands to Asia Minor.

In the practice of the present invention, the root of *Asphodelus Microcarpus* is utilized. In a specific embodiment of the present invention, the root or portions thereof may be administered to treat mammalian skin disorders. Preferably, in such a treatment, the outer skin of the root is first removed, and then the inner portion of the root is applied to the afflicted skin region. Additionally, raw extract from the roots may also be applied to the afflicted skin region.

It is to be understood in the practice of the present invention that the roots of *Asphodelus Microcarpus* may be harvested at any time. However, it is preferred that the roots be harvested at a time when they are full of liquid which for *Asphodelus Microcarpus* growing in Israel is generally from February to May.

Preparation of the *Asphodelus Microcarpus* roots is generally as follows. Excess dirt and other foreign matter should be removed from the roots, generally by shaking and water washing. After dirt and other foreign matter have been removed from the root, the next step is to remove the outer layer of the root. This can be accomplished by using a scraper, knife, a peeler such as a potato peeler, or the like.

The next step is to extract liquid from the roots. Methods for obtaining liquid from a compressible liquid-containing solid are well known to those of skill in the art, and any such method may be utilized. Simple methods include mashing, squeezing, pulverizing, liquefying, or compressing the roots. Preferably, the roots are liquefied in a commercially available "juicer."

The liquid thus obtained is mixed with acetic acid. Generally for this step, the volume ratio of *Asphodelus Microcarpus* root juice to acetic acid is in the range of about 1:20 to about 20:1. Preferably, the volume ratio is in the range of about 1:10 to about 10:1, more preferably in the range of about 1:5 to about 5:1, even more preferably approximately 4:1.

The *Asphodelus Microcarpus* root liquid is next mixed with acetic acid at any temperature suitable for creating a mixture of 3-methylanthralin, chrysophanol, aloe-emodin, and aloe-emodin monoacetate. It is preferable that the mixing occur with both the *Asphodelus Microcarpus* root liquid and acetic acid in the liquid state. Thus the contacting temperature is above the freezing point but below the boiling point for the mixture.

Optionally, in the practice of the present invention, brimstone may be mixed in with the *Asphodelus Microcarpus* root and acetic acid. The brimstone may be in any suitable form, but is preferably ground, and more preferably ground to a flour-like consistency.

The *Asphodelus Microcarpus* root/acetic acid mixture comprises 3-methylanthralin, chrysophanol, aloe-emodin, and aloe-emodin monoacetate and has been shown to be useful in the treatment of psoriasis. While this mixture is shown herein as being derivable from the *Asphodelus Microcarpus* root, it should be understood that the 3-methylanthralin, chrysophanol, and aloe-emodin can be obtained by the methods that are known in the art. Aloe-emodin monoacetate can be made by contacting aloe-emodin with acetic acid.

3-Methylanthralin, chrysophanol, aloe-emodin, and aloe-emodin monoacetate can be individually recovered from the mixture using separation techniques as are well known in the art.

The treatment composition of the present invention may include a wide range of the polyphenol components and/or their derivatives. In the method of the present invention for treating psoriasis, the composition may be administered to a mammalian organism by any route known in the art. Non-limiting examples of suitable routes of administration include oral, parenteral, topical, and the like. Specific non-limiting examples of carrying out such routes of administration include injection, IV administration, pills, tablets, capsules, liquids, gels, creams, soaps, shampoos, dermal patches, inhaled aerosols, sprays, suppositories, and the like. Topical administration is preferred for human psoriasis.

The frequency of administration varies with the strength of the composition, but an exemplary treatment schedule comprises a topical application once per day until symptoms are eradicated. The course of treatment naturally is dependent upon the severity of the affliction, and may last from 14 days up to 56 days, although this is not intended as limiting.

The present invention is described as being suitable for the treatment of humans for psoriasis, which is to be understood to include, but not be limited to, exfoliative psoriatic dermatitis, pustular psoriasis, and guttate variant psoriasis.

It is also believed that the compositions of the present invention are useful in the treatment of other skin disorders and conditions, including eczema.

In addition to being used in the treatment of skin disorders, the compositions of the present invention are also believed to be useful in the treatment of psoriatic arthritis.

EXAMPLES

The following examples are provided merely to illustrate the invention and are not intended to limit the scope of the invention in any manner.

Example 1

Obtaining Raw Extract

Raw extract was obtained from the *Asphodelus Microcarpus* plant as follows. Approximately 1 lb of roots of the plant were obtained from a location in Israel. Dirt and other foreign matter was removed from the roots by shaking, and then by washing with water, after which the outer skin of the roots was removed. Liquid was extracted from the peeled roots utilizing a commercially available "juicer". The 1 lb of roots yielded approximately 300 cc of raw extract.

Example 2

Preparation of Treatment Solution

Approximately 650 cc (although 400–800 cc are usable, depending upon the severity of the affliction) of the raw

extract obtained by the method of Example 1, approximately 350 cc (although 200–600 cc are usable) of acetic acid, and approximately 1 teaspoon of brimstone, ground to flour-like texture, were mixed together. This mixture does not require refrigeration, although the raw extract should be refrigerated until the acetic acid is introduced if kept in an unmixed state.

Example 3

Analysis of the Treatment Solution

The treatment solution of Example 2 was subjected to various types of chemical and physical analysis, the results of which are presented in Tables 1 and 2.

TABLE 1

Proximate Analysis Data*	
Physical description of sample	Dark brown liquid containing sediment and suspended particulate matter, acetic acid like odor
Ph (as received)	1.95
Acetic acid content (by titration)	5.05% w/w
Insoluble matter	2.14% w/w
Ash weight (mineral content)	0.746% w/w
Specific gravity (filtrate)	1.103 g/ml
Total dissolved solids (filtrate)	12.94% w/w
Chloroform extractables (filtrate) (organic soluble components)	0.84% w/w

*Analysis performed by Stillwell & Gladding Testing Laboratories, Inc., New York, N.Y.

TABLE 2

Elemental/Heavy Metal Composition*			
Element	Conc. ppm	Element	Conc. ppm
Sulfur	4260	Bismuth	ND < 3 ppm
Potassium	1640	Lead	ND < 3 ppm
Calcium	1075	Antimony	ND < 3 ppm
Sodium	216	Boron	ND < 2 ppm
Phosphorous	142	Indium	ND < 2 ppm
Magnesium	100	Molybdenum	ND < 1 ppm
Silicon	7	Arsenic	ND < 1 ppm
Iron	7	Selenium	ND < 1 ppm
Aluminum	5	Tellurium	ND < 1 ppm
Zinc	2	Thallium	ND < 1 ppm
Tin	1	Lithium	ND < 1 ppm
Strontium	0.9	Cobalt	ND < 1 ppm
Copper	0.9	Niobium	ND < 1 ppm
Manganese	0.4	Gallium	ND < 1 ppm
Titanium	0.4	Germanium	ND < 1 ppm
Nickel	0.4	Silver	ND < 1 ppm
Chromium	0.2	Cadmium	ND < .5 ppm
Barium	0.1	Mercury	ND < .5 ppm
Vanadium	0.06	Beryllium	ND < .1 ppm

*Elemental/heavy metal analysis was conducted by Umpire and Control Services, Inc., West Babylon, N.Y.

Example 4

Fractionation of Treatment Solution

The solution of Example 2 was also subjected to analysis. As the solution of Example 2 is too complex for direct analysis, it was fractionated according to the scheme in FIG. 1 using a combination of wet analytical chemistry, column chromatography and solvent extraction procedures. Throughout the analytical fractionation, isolates were sub-

jected to cell culture bioassay, as described in Example 6. Fractions testing positive in the cell culture bioassay were then directed to additional separation and/or chemical characterization until nearly pure isolates were obtained.

A 500 g sample 10 of the treatment solution of Example 2 was filtered through a Buchner funnel containing Whatman #1 filter paper and a 1 cm bed volume of Celite® analytical filtering aid. The filtrate 12 (485 g, 97% of original sample) tested positive in the cell culture bioassay and was equivalent in potency to the unfiltered treatment solution of Example 2. The filter retentate 14 (14.6 g, 2.92%) was a dark reddish brown colored material with a clay-like consistency. This substance tested negative in the cell culture bioassay. Analysis of this sediment indicates it to be largely composed of sucrose, complex carbohydrates, cellulosic debris, lignins, inorganic minerals, and clay.

The filtrate was then passed through a preconditioned chromatographic column packed with a 20×350 mm bed volume of XAD-2 resin. (Supelcopak-2® absorbent) at a flow rate of 5 ml/minute. XAD-2 resin is a hydrophobic porous polymer absorbent based on styrene-divinylbenzene copolymer. This resin has a high affinity for absorbing nonpolar, organic-soluble components from aqueous solutions. The column was preconditioned by washing it with 1.0 L of dichloromethane (DCM), followed by 1.0 L of methanol (MeOH) and finally 1.0 L of distilled/deionized water. All solvents were ultra-high-purity "capillary-analyzed" grade suitable for trace-level chemical analysis procedures. The distilled water used throughout the analysis was obtained from a Milli-Q® purification system. It was double distilled in glass and then further purified by passing it through ion-exchange and activated carbon filters. After the filtrate was passed through the XAD-2 resin, the column was washed with 2 L of distilled water. The filtrate and wash 15 from the XAD-2 resin column tested negative in the cell culture bioassay and were discarded. This dark brown colored fraction contains the water-soluble compounds of the treatment solution, such as acetic acid, sugars, low-molecular-weight polar organic acids, tannins, and other biologically inert components.

The XAD-2 resin column containing the treatment solution retentate 18 was blown dry to remove as much of the residual wash water as possible. The treatment solution organic-soluble components were then eluted from the column with 2.0 L of MeOH followed by 2.0 L of DCM. The MeOH and DCM XAD-2 resin eluates were collected separately. The treatment solution XAD-2 resin MeOH eluate 21 yielded 4.6 g, which corresponds to 0.92% of the original treatment solution on a weight basis. The eluate was amber colored. The treatment solution XAD-2 resin DCM eluate 23 contained 0.51 g or 0.1% of the original sample. This eluate was bright yellow colored. Both of these fractions tested positive in the cell culture bioassay. However, higher activity was observed in the treatment solution XAD-2 resin MeOH eluate. Therefore, this isolate was subjected to additional fractionation.

The treatment solution XAD-2 resin MeOH eluate 21 was concentrated to dryness in a round bottom flask using a rotary evaporator at reduced temperature and pressure. The residue was a dark red crystalline substance. The residue was dissolved in 200 ml of DCM, producing an amber-colored solution. However, not all the residue was soluble in DCM. The DCM-insoluble materials were then dissolved in 100 ml of MeOH. The methanol-soluble components had a dark red color. Thus two additional fractions were prepared. The treatment solution XAD-2 resin MeOH eluate DCM-soluble fraction 25 contained 1.7 g (0.34%), and the treat-

ment solution XAD-2 resin MeOH eluate DCM-insoluble fraction **26** yielded 2.9 g (0.58%). Both of these fractions tested positive in the cell culture bioassay.

The treatment solution XAD-2 resin MeOH eluate DCM-soluble fraction **40** contains organic-soluble neutral, phenolic, and acidic components. It was extracted 6 times with 100 ml portions of saturated sodium bicarbonate solution to remove the carboxylic acid fraction. The saturated sodium bicarbonate extract **28** was acidified to Ph 2.0 using 1 N HCL and then back-extracted 6 times with 50 ml portions of DCM to partition the carboxylic acids into the organic phase. The treatment solution carboxylic acid fraction **33** contained 166 mg (0.03%) and was weakly positive in the cell culture bioassay. The fraction had an amber-colored appearance.

The treatment solution XAD-2 resin MeOH eluate DCM-soluble fraction, after extraction of the carboxylic acids, contained 1.52 g or 0.30% of the original sample. This isolate **31** was named the treatment solution "neutral/phenol fraction" based on its chemical composition. It yielded a strong positive response in the cell culture bioassay. Upon refrigeration this fraction was observed to form a yellow-orange-colored crystalline precipitate. The precipitate was isolated by filtration through a sintered glass type Gooch crucible. The precipitate **36** isolated from the neutral/phenol fraction after extraction of acids yielded 186 mg (0.04%) of bright yellow-orange crystals. This isolate was found to be the most highly active fraction in the cell culture bioassay. The neutral/phenol fraction **38** after harvesting of the crystalline precipitate was found to contain 1.33 g (0.27%). This sample also tested strongly positive in the cell culture bioassay.

Some additional subfractions were prepared from the isolates described above. A portion of the treatment solution carboxylic acid fraction was methylated with diazomethane reagent to produce the corresponding methyl esters of the sample. This was done to enhance the volatility of the acids

to improve the gas chromatographic separation. The treatment solution XAD-2 resin MeOH eluate DCM-insoluble fraction was hydrolyzed with 1 N HCl for 4 hours at 100° C. to break down glycosidically conjugated species. The treatment solution neutral/phenol fraction **29** after harvesting of precipitate was subjected to additional minicolumn fractionation using a silica gel solid-phase extraction column. These procedures are described in more detail Example 5.

Example 5

Chemical Characterization of the Fractions of Example 4

The nonvolatile components of the fractions were analyzed by a combination of electron ionization direct probe mass spectrometry (DP data) and by a high-mass technique, liquid secondary ion mass spectrometry (LSIMS data).

The chemical composition of the XAD-2 resin DCM eluate fraction **23** is shown in Table 3, with peak assignments corresponding to the GC-MS chromatogram shown in FIG. 2. The DP and LSIMS spectra pertaining to the XAD-2 resin DCM eluate fraction **23** are shown in FIGS. 3 and 4, respectively. A homologous series of nonvolatile compounds with molecular weights 474, 490, 506, and 508 were detected in the DP data. Accurate mass measurements were performed on these peaks using high-resolution ($R=10,000$) mass spectrometry in order to determine their elemental formulas. The empirical formulas for these compounds were found to be $C_{23}H_{22}O_{11}$ (474 MW), $C_{23}H_{22}O_{12}$ (490 MW), $C_{13}H_{22}O_{13}$ (506 MW), and $C_{23}H_{24}O_{13}$ (508 MW). In other fractions related homologues with molecular weights 478 and 492 were also observed. Exact chemical structures for these compounds are unknown. However, their elemental formulas and mass spectral fragmentation patterns indicate that they are a class of polyphenolic chemical compounds called bisflavanoids.

TABLE 3

Chemical Composition XAD-2 Resin DCM Eluate Fraction 23 of FIG. 1			
MS Spec #	Compound (Synonyms, Common Names, Comments, Etc.)	CAS #	Peak Area %
66	phenol	108-95-2	1.43
282	sulfur (elemental sulfur six membered ring)	NA	0.90
372	dibutylphthalate (plasticizer)	84-74-2	0.29
441	hexadecanoic acid (palmitic acid)	57-10-3	0.11
445	sulfur (S-8, cyclic sulfur, orthorhombic sulfur, molecular sulfur)	10544-50-0	9.02
452	1-hexadecanol(hexadecyl alcohol)	36653-82-4	0.81
485	linoleic acid	60-33-3	0.91
519	3-methyl-1,8,9-anthracenetriol (3-methylanthralin, Chrysarobin)	491-59-8	5.86
527	1,8-dihydroxy-3-methyl-9,10-anthracenedione (Chrysophanol, Chrysophanic acid, 1,8-dihydroxy-3-methyl-1,8-anthraquinone)	481-74-3	11.76
549	1,8-dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione (Aloe-Emodin)	NA	0.14
574	2,4-bis(dimethylbenzyl)-6-t-butylphenol	NA	0.27
583	di-2-ethylhexylphthalate (plasticizer)	117-81-7	65.44
619-622	monoacetate derivative of 1,8-dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione (Aloe-Emodin monoacetate)	NA	2.23
DP Data	bis-flavanoids with m.w.'s 474 ($C_{23}H_{22}O_{11}$), 490 ($C_{23}H_{22}O_{12}$), 506 ($C_{13}H_{22}O_{13}$) and 508 ($C_{23}H_{24}O_{13}$) exact chemicals structures unknown	NA	1.73 (est. total for DP & LSIMS)

TABLE 3-continued

Chemical Composition XAD-2 Resin DCM Eluate Fraction 23 of FIG. 1			
MS Spec #	Compound (Synonyms, Common Names, Comments, Etc.)	CAS #	Peak Area %
LSIMS Data	unknown high mass compounds m.s. 608 & 696	NA	1.73 (est. total for DP & LSIMS data

DP = Data is from Electron Ionization Direct Insertion Probe
LSIMS = Liquid Secondary Ion Mass Spectrometry

The chemical composition of XAD-2 resin MeOH eluate DCM-insoluble fraction 26 is summarized in Table 4. The GC-MS chromatogram, DP data, and LSIMS data pertaining to this fraction are presented in FIGS. 5, 6 and 7. Most of this fraction was nonvolatile, and so the GC-MS peaks described make up only a small portion of this sample. The majority of the mass in this fraction consists of high-molecular-weight nonvolatile compounds. The DP data show relatively trace levels of several bisflavanoids. The LSIMS data show a complex mixture of high-mass compounds in the range 300–1000 to be present in this fraction. This fraction largely consists of “bound” compounds such as glycosides (phenolic compounds bound to sugar molecules) and other polar high-molecular-weight conjugates. This fraction was digested with HCl and heat in order to hydrolyze the conjugates down to small molecules, which could then be identified.

TABLE 4

Chemical Composition XAD-2 Resin MeOH Eluate DCM-Insoluble Fraction (Fraction 26 of FIG. 1)			
MS Spec #	Compound (Synonyms, Commons Names, Comments, Etc.)	CAS #	Peak Area %
423	palmitic acid	57-10-3	17.05
447	unk. 216 m.w. aromatic	NA	2.41
468	linoleic acid	60-33-3	28.08
519	3-methyl-1,8,9-anthracenetriol (3-methylanthralin, Chrysarobin)	491-59-8	27.82
527	1,8-dihydroxy-3-methyl-9,10- anthracenedione (Chrysophanol, Chrysophanic acid, 1,8- dihydroxy-3-methyl-1,8-anthraquinone)	481-74-3	12.78
583	di-2-ethylhexylphthalate (plasticizer)	117-81-7	11.87
DP	bis-flavanoids with m.w.'s 492	NA	trace
Data	(C ₂₃ H ₂₄ O ₁₂) and 506 (C ₂₃ H ₂₂ O ₁₃) exact chemicals structures unknown		
LSIMS	complex mixture of unknown compounds	NA	major
Data	m.w. range 300–1000 (bound components, glycosides, high m.w. polar conjugates etc.)		

DP = Data is from Electron Ionization Direct Insertion Probe raw data file FM10647
LSIMS = Liquid Secondary Ion Mass Spectrometry Data file VG2268

The data on the hydrolyzed XAD-2 resin MeOH eluate DCM-insoluble fraction 29 are summarized in Table 5. The GC-MS chromatogram, DP data, and LSIMS data for this fraction are shown in FIGS. 8, 9, and 10, respectively. Please note that the compounds in Table 3 that appear following hydrolysis are all present as bound components in the original fraction.

TABLE 5

Chemical Composition of Hydrolyzed XAD-2 Resin MeOH Eluate DCM-Insoluble (Fraction 29 of FIG. 1)			
MS Spec #	Compound (Synonyms, Common Names Comments, Etc.)	CAS #	Peak Area %
57	fumaric acid	110-17-8	4.14
97	2-ethyl-1-hexanol (plasticizer degradate)	104-76-7	1.8
123	2-furancarboxylic acid	88-14-2	2.64
151	4-oxo-pentanoic acid (levulinic acid)	123-76-2	5.36
183	α -hydroxyhexanoic acid	NA	0.62
271	branched dodecanol isomer	NA	1.56
278	1-chlorododecane (probably a hydrolysis artifact)	112-52-7	0.91
282	1-dodecanol (lauryl alcohol)	112-53-8	15.7
318	dodecanoic acid (lauric acid)	143-07-7	2.54
345	tributylphosphate (plasticizer)	126-73-8	1.29
348	1-tetradecanol (myristyl alcohol)	112-72-1	5.89
361	2-ethoxy-1-dodecanol	29718-44-3	7.09
365	2,8-dihydroxy-3-methyl-1,4- naphthoquinone (Droserone)	NA	3.29
378	tetradecanoic acid (myristic acid)	544-63-8	2.31
420	2-ethoxy-1-tetradecanol	NA	3.01
422	methyl palmitate	112-39-0	0.46
428	7-hydroxy-5-methoxy-2-methyl-4-oxo-4H- 1-benzopyran-6-carboxaldehyde	7338-51-4	1.15
441	hexadecanoic acid (palmitic acid)	57-10-3	7.54
440	α -hydroxylauric acid	NA	3.4
461	heptadecanoic acid (margaric acid)	506-12-7	0.32
449	oleic acid	112-80-1	0.69
480	linoleic acid	60-33-3	4.93
511	α -hydroxymyristic acid	2507-55-3	0.42
488	octadecanoic acid (stearic acid)	57-11-4	1.35
516	3-methyl-1,8,9-anthracenetriol (3-methylanthralin, Chrysarobin)	491-59-8	0.42
520	1,8-dihydroxy-3-methyl-9,10- anthracenedione (Chrysophanol, Chrysophanic acid, 1,8- dihydroxy-3-methyl-1,8-anthraquinone)	481-74-3	4.71
574	di-2-ethylhexylphthalate (plasticizer)	117-81-7	4.48
DP	bis-flavanoids with m.w.'s 478	NA	11.98
Data	(C ₂₂ H ₂₂ O ₁₂), 492 (C ₂₃ H ₂₄ O ₁₂) and 506 (C ₂₃ H ₂₂ O ₁₃) exact chemicals structures unknown		

DP = Data is from Electron Ionization Direct Insertion Probe raw data file FM10827

The carboxylic acid fraction 28 data are summarized in Table 6. The GC-MS chromatogram, DP data, and LSIMS data pertaining to this fraction 28 are presented in FIGS. 11, 12, and 13. Carboxylic acid fractions often contain nonvolatile species that do not readily pass through gas chromatography. These compounds can be chemically derivatized into more volatile forms using methylation or silylation reagents. Therefore, the carboxylic acid fraction 28 was methylated using freshly prepared diazomethane reagent. This procedure converts the carboxylic acids into their corresponding methyl esters. Phenols are converted into methyl ethers. The

methyl esters and methyl ethers are more volatile and chromatograph better than the free acids.

The GC-MS chromatogram from the methylated carboxylic acids is shown in FIG. 14. No additional compounds were detected in the methylated sample. All the same compounds observed in the underivatized carboxylic acid fraction were found as their methylated counterparts.

TABLE 6

Chemical Composition of Carboxylic Fraction (Fraction 28 FIG. 1)			
MS Spec #	Compound (Synonyms, Common Names, Comments, Etc.)	CAS #	Peak Area %
37-40	2-methylacrylic acid	79-41-4	14.4
49	2-methylbutyric acid	116-53-0	2.04
57	pentanoic acid	109-52-4	2.2
118	hexanoic acid	142-62-1	3.52
120	2-furancarboxylic acid	88-14-2	9.18
153	unknown 138 m.w. sulfur containing compound	NA	2.7
167	2-ethylhexanoic acid	149-57-5	1
181	benzoic acid	65-85-0	2.91
197	octanoic acid	123-07-2	6.29
217	benzeneacetic acid	103-82-2	1.2
227	nonanoic acid	112-05-0	4.74
242	decanoic acid	334-48-5	5.2
273	2-hydroxy-3-methylbenzoic acid (cresotic acid homosalicylic acid)	83-40-9	0.77
277	phenylpropenoic acid (cinnamic acid)	621-82-9	0.69
305	3,4-dichlorobenzoic acid	51-44-5	0.88
318	dodecanoic acid (lauric acid)	143-07-7	0.62
323	10-undecenoic acid	112-38-9	0.72
328	nonanedioic acid	123-38-9	0.54
329	pentachlorophenol (wood preservative)	87-86-5	0.11
342	3-methoxy-4-hydroxybenzaldehyde (vanillin)	121-33-5	0.75
372	p-coumaric acid, methyl ester	3943-97-3	1.23
378	tetradecanoic acid (myristic acid)	544-63-8	0.72
395	methyl ferulate	2309-07-1	0.72
408	methyl ester of 3,4-dimethoxycinnamic acid	5396-64-5	0.71
414	1,4-benzene dicarboxylic acid (terephthalic acid)	100-21-0	0.67
423	3,4-dihydro-4,8-dihydroxy-3-methyl-isocoumarin (6-hydroxymellain)	309-51-112	0.42
427	9-hexadecenoic acid (palmitoleic acid)	2091-29-4	0.55
434	hexadecanoic acid (palmitic acid)	57-10-3	1.9
438	9-octadecenoic acid (oleic acid)	112-80-1	0.49
478	9,12-octadecadienoic acid (linoleic acid)	60-33-3	1.03
484	octadecanoic acid (stearic acid)	57-11-4	0.69
491	3-methyl-1,8,9-anthracenetriol (3-methylanthralin, Chrysarobin)	491-59-8	0.55
516	1,8-dihydroxy-3-methyl-9,10-anthracenedione (Chrysophanol, Chrysophanic acid, 1,8-dihydroxy-3-methyl-1,8-anthraquinone)	481-74-3	1.3
572	di-2-ethylhexylphthalate (plasticizer)	117-81-7	0.6
633	squalene	7683-64-9	0.74
DP	bis-flavonoids with m.w.'s 492 (C ₂₃ H ₂₄ O ₁₂) and 506 (C ₂₃ H ₂₂ O ₁₃) extract chemicals structures unknown	NA	27.22
Data			est. total for OP & LSIMS
LSIMS	unknown high mass compounds m.w. 530, 654 and 740	NA	27.22
Data			est. total for DP & LSIMS

DP = Data is from Electron Ionization Direct Insertion Probe raw data file FM10648
LSIMS = Liquid Secondary Mass Spectrometry Data file VG2267

Table 7 summarizes the chemical composition of the yellow precipitate which was isolated from the neutral/phenol fraction 36 after extraction of the carboxylic acids. This fraction, which contains relatively few components,

possesses the highest degree of activity in the cell culture bioassay. The GC-MS chromatogram, DP data, and LSIMS data pertaining to this fraction are shown in FIGS. 15-17.

TABLE 7

Chemical Composition Precipitate Neutral/Phenol Fraction after Extraction of Acids (Fraction 36 of FIG 1)			
MS Spec #	Compound (Synonyms, Common Names Comments, Etc.)	CAS #	Peak Area %
419	methyl palmitate	112-39-0	0.53
464	methyl linoleate	112-63-0	1.09
510	3-methyl-1,8,9-anthracenetriol (3-methylanthralin, Chrysarobin)	491-59-8	19.69
517	1,8-dihydroxy-3-methyl-9,10- anthracenedione (Chrysophanol, Chrysophanic acid, 1,8- dihydroxy-3-methyl-1,8-anthraquinone)	481-74-3	57.41
583	d-2-ethylhexylphthalate (plasticizer)	117-81-7	18.35
DP	bis-flavanoids with m.w.'s 474	NA	2.93
Data	(C ₂₃ H ₂₂ O ₂₂), 492 (C ₂₃ H ₂₄ O ₁₂), 506 (C ₂₃ H ₂₂ O ₁₃) and 508 (C ₂₃ H ₂₄ O ₁₃) exact chemicals structures unknown		(est. total for DP & LSIMS)
LSIMS	unknown high mass compounds m.w. 618, NA		2.93
Data	662, 696, 718, 736 and 758		(est. total for DP & LSIMS data

DP = Data is from Electron Ionization Direct Insertion Probe raw data file
FM10645

LSIMS = Liquid Secondary Ion Mass Spectrometry Data file VG2270

The neutral/phenol fraction following extraction of carboxylic acids and harvesting of the yellow precipitate was still too complex for direct analysis. Therefore, it was subjected to additional fractionation using a silica gel solid phase extraction minicolumn procedure. The fraction was passed through the silica gel column and was washed with DCM. A second more polar fraction was then eluted from the column using methanol. Out of the original 1.32 g of neutral/phenol fraction were recovered 0.73 g (0.15% of original crude extract) in the DCM eluate and 0.59 g (0.12%) in the MeOH eluate. Therefore, the neutral/phenol fraction following extraction of carboxylic acids and harvesting of yellow precipitate was split into two additional subfractions for analysis. These were named the neutral/phenol fraction DCM silica gel eluate and the neutral/phenol fraction MEOH silica gel eluate. The chemical compositions of these two fractions are summarized in Tables 8 and 9. The GC-MS chromatograms, DP data, and LSIMS data pertaining to these two fractions are shown in FIGS. 17 through 22.

TABLE 8

Chemical Composition Neutral/Phenol Fraction Silica Gel MEOH Eluate			
MS Spec #	Compound (Synonyms, Common Names, Comments, Etc.)	CAS #	Peak Area %
134	3,5,5-trimethyl-2-cyclohexene-1-one (isophorone)	78-59-1	0.83
205- 225	5-hydroxymethylfurfuryl acetate (HMF acetate)	10551-58-3	6.24
231	glycerylmonoacetate	26446-35-5	1.03
244	glyceryldiacetate	25395-31-7	0.6
246	glyceryltriacetate (triacetin)	102-76-1	1
254	decanoic acid	334-48-5	0.18
295	1,4-butanediol diacetate	628-87-1	0.57
311	unknown aromatic acetate	NA	0.53
319	unknown aromatic acetate	NA	0.65
326	dodecanoic acid (lauric acid)	143-07-7	2.11
334	1,4-benzenediol monoacetate (hydroquinone monoacetate)	NA	2.03
353	unknown aromatic acetate	NA	0.29
359	unknown aromatic acetate	NA	0.13
372	myristic acid	544-63-8	2.66
399	unknown aromatic acetate	NA	0.5
405	pentadecanoic acid	1002-84-2	1.6
421	1,4-benzenedicarboxylic acid (terephthalic acid)	100-21-0	0.58
428	hexadecanoic acid (palmitic acid)	57-10-3	11.58
456	heptadecanoic acid	506-12-7	1.67
474	linoleic acid	60-33-3	2.88
500	octadecanoic acid (stearic acid)	57-11-4	39.02
520	3-methyl-1,8,9-anthracenetriol (3-methylanthralin, Chrysarobin)	491-59-8	2.57
529	1,8-dihydroxy-3-methyl-9,10-anthracenedione (Chrysophanol, Chrysophanic acid, 1,8-dihydroxy-3-methyl-1,8-anthraquinone)	481-74-3	5.7
539- 575	mixture of long chain aliphatic acetates	NA	4
583	di-2-ethylhexylphthalate (plasticizer)	117-81-7	0.55
616	1,8-dihydroxy-3-(hydroxymethyl)-9,10- anthracenedione (Aloe-Emodin)	NA	3.82

TABLE 8-continued

Chemical Composition Neutral/Phenol Fraction Silica Gel MEOH Eluate			
MS Spec #	Compound (Synonyms, Common Names, Comments, Etc.)	CAS #	Peak Area %
622	monoacetate derivative of 1,8-dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione (Aloe-Emodin monoacetate)	NA	1.34
636	unknown 378 m.w. aromatic acetate	NA	0.77
644	squalene	7683-64-9	0.27
DP	bis-flavanoids with m w 's 474 (C ₂₃ H ₂₂ O ₁₁), 478 (C ₂₃ H ₂₂ O ₁₂), 490 (C ₂₃ H ₂₂ O ₁₂), 492 (C ₂₂ H ₂₄ O ₁₂), 506 (C ₂₃ H ₂₂ O ₁₃) and 508 (C ₂₃ H ₂₄ O ₁₃) exact chemicals structures unknown	NA	4.3 (est. total for DP & LSIMS)
LSIMS Data	unknown high mass compounds m.s. 608, 680 and 696	NA	4.3 (est. total for DP & LSIMS data)

DP = Data is from Electron Ionization Direct Insertion Probe raw data file FM10650

LSIMS = Liquid Secondary Ion Mass Spectrometry Data file VG2265

TABLE 9

Chemical Composition Neutral/Phenol Fraction Silica Gel DCM Eluate			
MS Spec #	Compound (Synonyms, Common Names, Comments, Etc.)	CAS #	Peak Area %
263	unknown 154 m.w. compound	NA	0.29
268	2,3-dihydro-2,5-dimethyl-4H-1-benzopyran-4-one	69687-87-2	0.88
278	S-6 hexasulfide (six membered ring structure)	NA	1.35
283	2,6-di-t-butyl-p-hydroxyanisole (antioxidant)	NA	2.12
295	butylated hydroxy toluene (BHT, antioxidant)	128-37-9	3.65
301	methyl laurate	111-82-0	1.47
323	diethylphthalate (plasticizer)	84-66-2	1.06
329	dodecyl acetate	112-66-3	1.82
358	unknown benzoate	NA	0.65
365	methyl tridecanoate	1731-88-0	1.35
369	α -hexylcinnamic aldehyde	101-86-0	0.65
373	benzyl benzoate	120-51-4	0.71
386	methylpentadecanoate	7132-64-1	0.82
390	tetradecyl acetate	638-59-5	0.59
404	benzyl salicylate	118-58-1	1.35
417	acetylferulic acid	2596-47-6	2.12
425	methyl plamitate	112-39-0	4.53
433	dibutylphthalate	84-74-2	2.00
441	sulfur (S-8, cyclic sulfur, orthorhombic sulfur, molecular sulfur)	10544-50-0	3.59
447	hexadecyl acetate	629-70-9	1.88
451	methyl heptadecanoate	1731-92-6	0.94
471	methyl linoleate	112-63-0	15.82
478	methyl stearate	112-61-8	0.76
515	3-methyl-1,8,9-anthracenetriol (3-methylanthralin, Chrysarobin)	491-59-8	5.06
519-533	1,8-dihydroxy-3-methyl-9,10-anthracenedione (Chrysophanol, chrysophanic acid, 1,8-dihydroxy-3-methyl-1,8-anthraquinone)	481-74-3	15.00
543	di-2-ethylhexylphthalate (plasticizer)	117-81-7	12.53
617	monoacetate derivative of 1,8-dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione (Aloe-Emodin monoacetate)	NA	6.24
639	methyl farnesate	10485-70-8	1.06
DP Data	bis-flavanoids with m.w.'s 474 (C ₂₃ H ₂₂ O ₁₁), 490 (C ₂₃ H ₂₂ O ₁₂), 492 (C ₂₃ H ₂₄ O ₁₂), 508 (C ₂₃ H ₂₂ O ₁₃) and 508 (C ₂₃ H ₂₄ O ₁₃) exact chemicals structures unknown, also unknown peaks at m w. 570 and 602	NA	9.71 (est. total for DP & LSIMS)

TABLE 9-continued

Chemical Composition Neutral/Phenol Fraction Silica Gel DCM Eluate			
MS Spec #	Compound (Synonyms, Common Names, Comments, Etc.)	CAS #	Peak Area %
LSIMS Data	unknown high mass compounds m.w. 540, 616, 646 and 662	NA	9.71 (est. total for DP & LSIMS data)

DP = Data is from Electron Ionization Direct Insertion Probe raw data file FM10649
 LSIMS = Liquid Secondary Ion Mass Spectrometry Data file VG2266

The data obtained from all of the fractions of Example 4 were pooled together and normalized to produce a comprehensive summary. These data are summarized in Tables 10A-F, which describe the chemical composition of the organic soluble fraction, which constitutes approximately 1% of the treatment solution on a w/w basis but contains 100% of the biological activity. The compounds in this table are grouped together by chemical class. It should be noted that the quantitative data (% w/w) provided in this and the other tables are not exact but rather are semiquantitative. The data were derived from a combination of GC area % integrations and from gravimetric determinations made throughout the fractionation. Highly accurate quantitation data would only be possible if analytical reference standards were available for all compounds detected so that detector response factors could be determined and the data adjusted.

TABLE 10A

Data Summary: Composition of Organic Soluble Fraction Grouped by Chemical Class		
Aliphatic Carboxylic Acids	CAS #	% w/w
2-methylacrylic acid	79-41-4	0.27
2-methylbutyric acid	116-53-0	0.04
fumaric acid	110-17-8	1.50
pentanoic acid	109-524	0.04
hexanoic acid	142-62-1	0.07
2-furanocarboxylic acid	88-14-2	1.13
2-ethylhexanoic acid	149-57-5	0.02
4-oxo-pentanoic acid (levulinic acid)	123-76-2	1.94
α -hydroxyhexanoic acid	NA	0.23
octanoic acid	124-07-2	0.118
nonanoic acid	112-05-0	0.09
decanoic acid	33448-5	0.11
dodecanoic acid (lauric acid)	143-07-7	1.09
10-undecenoic acid	112-38-9	0.01
nonanedioic acid	123-38-9	0.01
tetradecanoic acid (myristic acid)	544-63-8	1.05
pentadecanoic acid	1002084-2	0.12
hexadecanoic acid (palmitic acid)	57-10-3	9.82
α -hydroxylauric acid	NA	1.232
α -hydroxymyristic acid	2507-55-3	0.15
heptadecanoic acid	506-12-7	0.24
oleic acid	112-80-1	0.259
linoleic acid	60-33-3	12.26
stearic acid	57-11-4	3.43
palmitoleic acid	2091-29-4	0.01

TABLE 10B

Aromatic Carboxylic Acids		
benzoic acid	65-85-0	0.06
benzeneacetic acid	103-82-2	0.02
3,4-dichlorobenzoic acid	51-44-5	0.02

TABLE 10B-continued

Aromatic Carboxylic Acids		
1,4-benzenedicarboxylic acid (terephthalic acid)	100-21-0	0.06
cinnamic acid	621-82-9	0.01
acetyl ferulic acid	2596-47-6	0.20

TABLE 10C

Esters		
glyceryl monoacetate	26446-35-5	0.08
glyceryl diacetate	25395-31-7	0.04
glyceryl triacetate (triacetin)	102-76-1	0.08
1,4-butanediol diacetate	628-67-1	0.04
2-hydroxy-5-methylfurfuryl acetate (HMF acetate)	10551-58-3	0.47
diethylphthalate (plasticizer)	84-66-2	0.10
dibutylphthalate (plasticizer)	84-74-2	0.21
di-2-ethylhexylphthalate (plasticizer)	117-81-7	11.70
tributylphosphate (plasticizer)	126-73-8	0.47
methyl laurate	111-82-0	0.14
methyl tridecanoate	1731-88-0	0.13
methyl pentadecanoate	7132-64-1	0.08
methyl palmitate	112-39-0	0.60
methyl heptadecanoate	1731-92-6	0.09
methyl linoleate	112-63-0	1.51
methyl stearate	112-61-8	0.07
methyl farnesate	10485-70-8	0.10
dodecyl acetate	112-66-3	0.17
tetradecyl acetate	638-59-5	0.06
hexadecylacetate	629-70-9	0.18
benzyl benzoate	120-51-4	0.54
benzyl salicylate	118-58-1	0.13
methyl ester of 3,4-dimethoxy cinnamic acid	5396-64-5	0.01
unknown aromatic acetates	NA	0.16
mixture of long chain aliphatic acetates	NA	0.30
unknown benzoate	NA	0.06
unknown 378 m.w. aromatic acetate	NA	0.06

TABLE 10D

Phenolic Compounds		
phenol	108-95-2	0.09
2-hydroxy-3-methylbenzoic acid (cresotic acid, homosalicylic acid)	83-40-9	0.01
ferulic acid methyl ester	2309-07-1	0.01
benzyl salicylate	118-58-1	0.13
3,4-dihydro-4,8-dihydroxy-3-methylisocoumarin (6-hydroxymellein)	309-51-112	0.01
7-hydroxy-5-methoxy-2-methyl-4-oxo-4H-1-benzopyran-6-carboxaldehyde	7338-51-4	0.42
2,6-di-t-butyl-4-methylphenol (BHT, antioxidant)	128-37-0	0.34
2,6-di-t-butyl-p-methylanisole (antioxidant)	NA	0.20
2,4-bis-(dimethylbenzyl)-6-t-butylphenol (antioxidant)	NA	0.02

TABLE 10D-continued

Phenolic Compounds		
hydroquinone monoacetate	NA	0.15
4-hydroxy-3-methoxybenzaldehyde (vanillin)	121-33-5	0.01
p-coumaric acid methyl ester	3943-97-3	0.02
pentachlorophenol (PCP, wood preservative)	87-86-5	0.002
3-methyl-1,8,9-anthracenetriol	491-59-8	11.77
(3-methylanthralin, Chrysarobin)		
18-dihydroxy-3-methyl-9,10-anthracenedione	481-74-3	10.37
(Chrysophanol, Chrysophanic acid, 1,8-dihydroxy-3-methyl-1,8-anthraquinone)		
1,8-dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione (Aloe-Emodin)	481-72-1	0.30
monoacetate derivative of 1,8-dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione (Aloe-Emodin monoacetate)	NA	0.83
2,8-dihydroxy-3-methyl-1,4-naphthoquinone (Droserone)	NA	1.19
bis-flavanoids with m.w.'s 474 (C ₂₂ H ₂₂ O ₁₁), 478 (C ₂₂ H ₂₂ O ₁₂), 490 (C ₂₃ H ₂₂ O ₁₂), 492 (C ₂₃ H ₂₄ O ₁₂), 506 (C ₂₃ H ₂₂ O ₁₃) and 508 (C ₂₃ H ₂₄ O ₁₃) exact chemical structures unknown	NA	6.27 est. total for DP & LSIMS

TABLE 10E

Alcohols		
2-ethyl-1-hexanol	104-76-7	0.65
branched dodecanol isomer	NA	0.57
1-dodecanol (dodecylalcohol)	112-53-8	5.69
1-tetradecanol (tetradecyl alcohol)	112-72-1	2.14
2-ethoxy-1-dodecanol	29718-44-3	2.57
1-hexadecanol (hexadecyl alcohol)	36653-82-4	0.05
2-ethoxy-1-tetradecanol	NA	1.09

TABLE 10F

Miscellaneous Compounds		
3,5,5-trimethyl-2-cyclohexene-1-one (isophorone)	78-59-1	0.06
2,3-dihydro-2,5-dimethyl-4H-1-benzopyran-4-one	69687-87-2	0.08
α -hexylcinnamic aldehyde	101-86-0	0.06
squalene	7683-64-9	0.03
1-chloro-dodecane (dodecyl chloride)	112-52-7	0.33
sulfur (elemental sulfur six membered ring)	NA	0.18
sulfur (S-8, cyclic sulfur, orthorhombic sulfur, molecular sulfur)	10544-50-0	0.90
unknown 138 m.w. sulfur-containing compound	NA	0.05
unknown 216 m.w. aromatic compound	NA	0.87
unknown 154 m.w. aromatic compound	NA	0.03
complex mixture of high molecular weight	NA	6.27 est.

TABLE 10F-continued

Miscellaneous Compounds		
5	unknown compounds in the range 400-1000, many of these compounds are conjugates of the compounds identified in this study such as glycosides, polar conjugates, high m.w. esters etc.	total for DP & LSIMS

Example 6

In vivo Study. Application of the Treatment Solution to a Flaky Skin Mouse

The flaky skin mouse (fsn) is a genetically engineered mouse with an autosomal recessive mutation causing the skin to resemble that exhibited in human psoriasis (see Sundberg et al., *J. Vet. Diagn. Invest.* 4:312-17, 1992).

In the homozygous affected flaky skin mouse (fsn/fsn), where the mutation is on both chromosomes, histological features such as marked acanthosis, hyperkeratosis with focal parakeratosis, subcorneal pustules, dermal capillary dilation, and dermal infiltration of inflammatory cells are seen.

Ten affected mice (fsn/fsn) and 10 normal littermate controls (fsn/-) were obtained from The Jackson Laboratory in Bar Harbor, Me. The animals were maintained using standard diet and housing.

The dorsal surface of each animal was shaved. One-half of the dorsal surface received weekly topical treatments of the solution of Example 2, while the other half served as an untreated control. The solution of Example 2 was applied to the skin using a sterile cotton swab. Treatments continued for up to seven weeks. Weekly biopsies of treated and untreated areas were also removed for histological examination. Following the seventh week of treatment, the animals were necropsied for gross pathological changes.

The solution of Example 2 was found to induce dramatic gross and microscopic changes only for the hyperproliferative skin of the affected (fsn/fsn) mice with minimal to no effects noted for the (fsn/-) control mice. Observations by a veterinary dermatopathologist described the skin response of the affected animals as being similar to a "chemical burn." Histologic findings indicated that the keratinocyte growth of the affected mouse skin was markedly reduced, and that the treated skin began to slough off a bit but remained fully attached as a biological bandage allowing healing of the underlying skin. This effect was not observed in the control animals or untreated skin in the affected animals. Results are presented in Table 11.

TABLE 11

Summary of Flaky Skin Mouse Study				
I.D. NUMBER	Phenotype	Histological Assessment	Gross Pathologic Changes	Comments
1 (fsn/fsn)	Flaky skin	Reduction in proliferation of keratinocytes	None observed	
2 (fsn/fsn)	Flaky skin	Reduction in proliferation of keratinocytes	None observed	
3 (fsn/fsn)	Flaky skin	Reduction in proliferation of	None observed	

TABLE 11-continued

Summary of Flaky Skin Mouse Study				
I D. NUMBER	Phenotype	Histological Assessment	Gross Pathologic Changes	Comments
4 (fsn/fsn)	Flaky skin	keratinocytes Reduction in proliferation of keratinocytes	None observed	
5 (fsn/fsn)	Flaky skin	Reduction in proliferation of keratinocytes	None observed	
6 (fsn/fsn)	Flaky skin	Reduction in proliferation of keratinocytes	None observed	
7 (fsn/fsn)	Flaky skin	Reduction in proliferation of keratinocytes	None observed	
8 (fsn/fsn)	Flaky skin	Reduction in proliferation of keratinocytes	None observed	
9 (fsn/fsn)	Flaky skin	Reduction in proliferation of keratinocytes	None observed	
10 (fsn/fsn)	Confusing phenotype	Marginal change	None observed	Jackson Labs suggest mistyping of animal
11 (+/-)	Normal skin	No change	None observed	
12 (+/-)	Normal skin	No change	None observed	
13 (+/-)	Normal skin	No change	None observed	
14 (+/-)	Normal skin	No change	None observed	
15 (+/-)	Normal skin	No change	None observed	
16 (+/-)	Normal skin	No change	None observed	
17 (+/-)	Normal skin	No change	None observed	
18 (+/-)	Normal skin	No change	None observed	
19 (+/-)	Normal skin	No change	None observed	
20 (+/-)	Normal skin	No change	None observed	

Example 7

Bioassay—In viva Study Bioassay

The in vivo study of Example 6 using the flaky skin mouse model was indicative of keratinocyte cell specifically with respect to the activity of the treatment solution compound.

In vitro cell testing is necessary to detect activity with respect to cell type for purposes of developing a bioassay for treatment solution activity, and second to further study results obtained using the flaky skin mice of Example 6. Because human skin contains both keratinocytes and fibroblasts, either of which may be involved in the psoriasis disease process, pure cultures of human fibroblasts were grown from normal adult human skin, keloid scars (hyperproliferative fibroblasts), and commercially available certified pure cultures of normal human adult epidermal keratinocytes. These cell types were tested separately for their growth responses to treatment with the treatment solution of Example 2.

Protocol

Cell growth is measured by examining the amounts of DNA synthesis, for as cells grow and divide, more DNA is produced. Radioactive thymidine is added to the cell culture media. The assay is conducted as follows: Cultured fibroblasts are grown in complete minimal essential medium (CMEM) containing 10% fetal bovine serum as a growth factor source. Epidermal keratinocytes were cultured in the presence of complete keratinocyte growth medium (CKGM) supplemented with bovine pituitary extract, hydrocortisone, and epidermal growth factor (EGF) as a growth factor source.

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Healthy growing cells were seeded into Corning 24 well tissue culture plates at a density of 1.0×10^4 cells per well in 1.0 ml of either CMEM or CKGM depending on cell type. The cells were incubated for 24–36 hours or until they reached 60–70% confluency at 37° C. in the presence of 5% CO₂. The medium was then changed to either MEM without the 10% serum or KGM without hydrocortisone or ECF, but leaving the BPE in the media. This allows starvation of the cells, or their regression to a nongrowth phase where although they are not dividing, they remain biochemically active. The cells were allowed to incubate under these conditions for 24 hours. The starving media were then removed and replaced with 1.0 ml/well of complete growth media CMEM or CKGM containing no treatment, treatment solution of Example 2, or a subtraction from Example 4 at a dilution of 1:5000, or solvent alone such as acetic acid or DMSO. Statistically significant numbers of repetitions were performed for each treatment. Additionally 1 μ Ci tritiated thymidine (³H) was also added per well. Following these treatments, the cells were allowed to incubate for 24 hours using identical conditions as above. Following the end of the incubation period, the wells were washed 3 times with phosphate buffered saline (PBS) and fixed with 12.5% trichloroacetic acid (TCA) for 10 minutes followed by methanol for 10 minutes. The plates were air dried and the cells solubilized in 1.0 ml of 0.2 N NaOH at 37° C. for 1 hour. Growth was determined by measuring the level of radioactivity present. This was accomplished by counting 0.9 ml of the solubilized cells in a scintillation counter.

55

Results

Fibroblasts

Fibroblasts from normal adult skin and keloid scar were cultured and assayed for effects of the treatment solution on

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60

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Keratinocytes

Tables 12–14 summarize the percentages of the 4 main active ingredients in each of those fractions, and mean percentages of inhibition in the keratinocyte bioassay for each fraction. Finally, mean percentages of inhibition are normalized to the percentages of each active ingredient in the fractions mentioned above.

Percentages of 4 Main Active Ingredients in the Treatment Solution of Example 2, Compiled from Tables 3-10					
Fraction	FIG. 1	3-methyl anthralin	chrysarobi	aloe- emodin	aloe-emodin monoacetate
	REF. NO.				
XAD-2 Resin DCM Eluate	23	5.86%	11.76%	0.14%	2.23%
XAD-2 Resin MeOH Eluate-DCM- Insol.	26	27.82%	12.78%	0%	0

Percentages of 4 Main Active Ingredients
in the Treatment Solution of Example 2,
Compiled from Tables 3-10

		FIG. 1				
	Fraction	REF. NO.	3-methyl anthralin	chrysarobi	aloe-emodin	aloe-emodin monoacetate
10	Hydrolyzed Z-92 XAD	29	0.42%	4.71%	0%	0%
15	Resin-MeOH Eluate					
	DCM Insol. Z-92	28	0.55%	1.3%	0%	0%
20	Carboxylic Acid Fx					
	Precipitate From Neutral/Phenol Fx	36	19.69%	57.41%	0%	0%
25	Neutral/Phenol Silica Gel MeOH Eluate	31	2.57%	5.7%	3.82%	1.34%
30	Neutral/Phenol Silica Gel DCM Eluate	31	5.06%	15.00%	0%	6.24%
	Crude Z-92	10	11.77%	10.37%	0.30%	0.83%

Fraction Analysis Summary Results -
% Growth Inhibition of Keratinocytes by Fraction

[illegible]